The mouse gene for hypoxia-inducible factor-1alpha. Genomic organization, expression and characterization of an alternative first exon and 5' flanking sequence

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Abstract: The ubiquitously expressed hypoxia-inducible factor-1 (HIF-1) is involved in expression of a large number of oxygen-regulated genes. HIF-1 is a heterodimer consisting of an alpha and a beta subunit, both belonging to the basic-helix-loop-helix Per-aryl hydrocarbon receptor nuclear translocator-Sim (PAS) family of transcription factors. Whereas HIF-1alpha is a novel member of this family, HIF-1beta is identical to the aryl hydrocarbon receptor nuclear translocator, previously recognized to be involved in xenobiotic metabolism. cDNA cloning revealed that mouse HIF-1alpha can be expressed as two mRNA isoforms containing alternative 5' untranslated regions and two different predicted translational start sites. We cloned and characterized 20.5 kb of the mouse HIF-1alpha gene (Hif1a) containing exon II-XV. The two alternative first exons, I.1 and I.2, are separated from exon II by approximately 24 kb and 17 kb, respectively. We also sequenced Hif1a exon I.1 and flanking regions, and mapped a single exon I.1 transcription initiation site. Reverse transcription PCR analysis of total RNA derived from normoxic and hypoxic mouse hepatoma and fibroblast cell lines suggested that the two alternative mRNA isoforms are constitutively coexpressed in these cells, and that two different promoters drive transcription of HIF-1alpha. A minimal exon I.1 promoter was identified which moderately activated heterologous gene expression, indicating that additional cis-elements are required for efficient HIF-1alpha transcription in vivo.

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The mouse gene for hypoxia-inducible factor-1α

Genomic organization, expression and characterization of an alternative first exon and 5′ flanking sequence

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The ubiquitously expressed hypoxia-inducible factor-1 (HIF-1) is involved in expression of a large number of oxygen-regulated genes. HIF-1 is a heterodimer consisting of an α and a β subunit, both belonging to the basic-helix-loop-helix Per-aryl hydrocarbon receptor nuclear translocator-Sim (PAS) family of transcription factors. Whereas HIF-1α is a novel member of this family, HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator, previously recognized to be involved in xenobiotic metabolism. cDNA cloning revealed that mouse HIF-1α can be expressed as two mRNA isoforms containing alternative 5′ untranslated regions and two different predicted translational start sites. We cloned and characterized 20.5 kb of the mouse HIF-1α gene (*Hif1a*) containing exon II–XV. The two alternative first exons, I.1 and I.2, are separated from exon II by approximately 24 kb and 17 kb, respectively. We also sequenced *Hif1a* exon I.1 and flanking regions, and mapped a single exon I.1 transcription initiation site. Reverse transcription PCR analysis of total RNA derived from normoxic and hypoxic mouse hepatoma and fibroblast cell lines suggested that the two alternative mRNA isoforms are constitutively co-expressed in these cells, and that two different promoters drive transcription of HIF-1α. A minimal exon I.1 promoter was identified which moderately activated heterologous gene expression, indicating that additional *cis*-elements are required for efficient HIF-1α transcription *in vivo*.

Keywords: erythropoietin; gene expression; hypoxia; promoter; transcription.

Mammalian organisms have developed multiple protective mechanisms to adapt to reduced oxygen concentrations (hypoxia) at the systemic, local and cellular levels. Adaptation to acute systemic hypoxia is achieved by increasing ventilation, which is triggered by the carotid body. Prolonged hypoxia upregulates the synthesis of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis in the carotid body. Adaptation to chronic systemic hypoxia also includes an increase in the oxygen-transport capacity by enhanced erythropoiesis which is dependent on the erythropoietin concentration in the blood. Expression of both tyrosine hydroxylase and erythropoietin is regulated directly as a function of the oxygen concentration at the levels of transcription and mRNA stability (reviewed in [1–4]). Vascular endothelial growth factor (VEGF) provides an example of adaptation to local hypoxia. By inducing angiogenesis, VEGF induction leads to increased vascularization and, hence, to decreased oxygen diffusion distances [5]. At the cellular level, hypoxia induces a switch from oxidative phosphorylation to glycolysis as the main source of ATP production. As a consequence, the expression of glucose transporters [6–8] and glycolytic enzymes [9–12] is increased.

At all of these levels, the signalling pathways transmitting the signal from an operationally defined putative heme oxygen sensor to the nucleus seem to share the transcription factor hypoxia-inducible factor 1 (HIF-1) (reviewed in [2–4, 13]). HIF-1 was originally detected as a factor binding to an element in the erythropoietin 3′ flanking region that is critically involved in hypoxic activation of erythropoietin gene transcription [14], and has subsequently been demonstrated to be involved in hypoxic regulation of all the genes mentioned above [7, 9–12, 15–19]. In addition, HIF-1 has also been implicated in oxygen-regulated expression of the genes encoding inducible nitric oxide synthase [20], VL30 retrotransposon [21] and transferrin (Rolfs, A., Kvietikova, I., Gassmann, M. and Wenger, R. H., unpublished results) and, therefore, it appears that HIF-1 functions as a key regulator of oxygen-dependent gene expression. The widespread nature of HIF-1 expression and function has been confirmed by reporter gene experiments and DNA-binding studies in many different mammalian cell lines [22, 23] and recently, an HIF-1-like activity has even been detected in insect cells [24].

Taking advantage of the DNA-binding properties of HIF-1, it was possible to purify the two subunits which constitute human HIF-1 [25], and subsequently also to clone and sequence them [26]. Both subunits (termed HIF-1α and HIF-1β) belong to a subfamily of basic-helix-loop-helix (bHLH) transcription factors which share a region of homology termed PAS (reviewed

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Abbreviations. AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; bHLH, basic-helix-loop-helix; EMSA, electrophoretic mobility shift assay; HIF, hypoxia-inducible factor; PAS, Per-ARNT-Sim; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; UTR, untranslated region; VEGF, vascular endothelial growth factor.

Note. The novel nucleotide sequences reported in this paper have been deposited with the EMBL/GenBank/DDBJ data bases and are available under accession numbers Y09085 and Y09086.

in [27, 28]). PAS stands for the prototype members of this family: the *Drosophila* proteins period (Per) and single minded (Sim), and the heterodimeric, dioxin-activated complex aryl hydrocarbon receptor (AhR)/aryl hydrocarbon receptor nuclear translocator (ARNT). Other members of the PAS family include the *Drosophila* proteins trachealess (Trh) [29, 30] and similar (Sima) [31], mouse and rat ARNT2 [32, 33] as well as human and rodent Sim homologs which may be involved in the pathogenesis of Down syndrome [34–39]. Whereas HIF-1α is a new member of the bHLH-PAS family, HIF-1β is identical to ARNT. Thus, ARNT is capable of heterodimerizing either with AhR or HIF-1α, followed by transactivation of genes involved in xenobiotic metabolism or adaptation to hypoxia, respectively [40–43]. The HIF-1 complex is exponentially induced over a physiologically relevant range of O_2 tension [44]. The mechanisms by which hypoxia activates HIF-1, however, have only partially been unravelled and are a matter of intense investigations. While, in some experimental settings, hypoxia increases HIF-1α (and in part also HIF-1β) mRNA levels [26, 45], we and others did not find increased HIF-1α mRNA levels following hypoxic exposure [40, 46–48]. Lack of increased mRNA levels suggests that HIF-1α is regulated by translational and/or post-translational mechanisms including protein stability [48], phosphorylation [49], redox modifications [50] or, as has been shown for AhR, retardation in the cytosolic compartment by complex formation with hsp90 [40].

Recently, we [46] and others [19] cloned the mouse HIF-1α cDNA which is 90% similar to the human HIF-1α cDNA. Mouse HIF-1α is encoded by an evolutionary conserved single-copy gene (designated *Hif1a*) which has been assigned to a region of conserved synteny on human chromosome 14q and mouse chromosome 12 [46, 51]. The most intriguing difference between the mouse HIF-1α cDNA cloned in our laboratory [46] and the cDNA reported by Li et al. [19] is the presence of an alternative 5′ untranslated region (UTR), which predicts the use of different ATG translation initiation codons [46]. Whereas the 5′ UTR of the mouse HIF-1α cDNA reported by Li et al. [19] is homologous to the previously cloned human cDNA [26], no human HIF-1α cDNA containing the alternative 5′ UTR has so far been reported. In this paper, we have cloned and characterized the mouse *Hif1a* gene, including the first exon encoding the alternative 5′ UTR, and show that mouse *Hif1a* is expressed as two mRNA isoforms containing two different 5′ UTRs.

MATERIALS AND METHODS

Cloning and sequencing of the mouse *Hif1a* gene. The mouse *Hif1a* gene was cloned from a genomic λ phage library (kind gift of U. Müller, Zürich, Switzerland) prepared from DNA which had been isolated from the mouse strain 129Sv(ev)-derived embryonic stem cell line AB-1, partially digested with Sau3AI and ligated into the vector LambdaGEM-11 (Promega). This library was screened by plaque hybridization [52] to a 0.5-kb *Hin*dIII 5′ terminal fragment from the human HIF-1α cDNA-bearing plasmid pBluescriptSK/HIF-1α3.2-3 [26] labeled by random-primed labeling with [α-^{32}P]dCTP (Hartmann). To obtain the entire coding region, the library was further screened with probes derived from the 3′ and 5′ UTRs of HIF-1α. The 3′ UTR probe consisted of a subcloned 0.3-kb PCR fragment amplified from mouse RNA using the human forward and reverse primers 5′-TGGCATTTATTTGGATAAA-3′ and 5′-TAGCGACAAAGTGCATAA-3′, respectively. The probe for the alternative 5′ UTR [46] was a 23-nucleotide synthetic oligonucleotide termed mHIFpex (5′-CACGGTAACAGTTTGCCCAGAAA-3′), complementary to the HIF-1α cDNA at positions 87–109 [46] (see also Fig. 3). The probe for the 5′ UTR homologous to human HIF-1α [19] was a 270-bp *Eco*RI–*Nco*I cDNA fragment that was kindly provided by A. Damert (Bad Nauheim, Germany). Positive λ clones were plaque purified, analyzed by restriction digestion and Southern blotting and subcloned into pBluescript vectors (Stratagene). The *Hif1a* gene was sequenced on both strands using a combination of various automated and manual sequencing procedures with fluorescently labeled dideoxynucleotides, fluorescently labeled primers and [^{35}S]dATP[αS] incorporation in cycle-sequencing reactions and T7-sequencing reactions, respectively, according to the instructions provided by the manufacturers (Applied Biosystems and Pharmacia).

Mung bean nuclease protection. A radioactively labeled probe was prepared by phosphorylation of the 5′ end of the oligonucleotide mHIFpex (see above) with [γ-^{32}P]ATP (Hartmann) and T4 polynucleotide kinase (Fermentas) as described elsewhere [53]. Circular, single-stranded DNA was obtained from the plasmid pH13X8ΔH using M13K07 helper phages [52]. This plasmid contained the 1374-bp *Xba*I–*Hin*dIII fragment which includes exon I.1 and 0.9 kb of upstream sequence (see also Fig. 3). The labeled mHIFpex primer was annealed to single-stranded pH13X8ΔH in 10 mM Tris/Cl, pH 8.0, 10 mM $MgCl_2$ for 15 min at 4°C, and extended by adding 1 mM dNTPs and Klenow fragment of DNA polymerase I (Fermentas) for 30 min at 37°C. Following heat inactivation (5 min at 70°C), the products were cleaved with *Bam*HI (Fermentas) and ethanol precipitated. The resulting 523-bp probe was denatured in 30 mM NaOH and the single-stranded probe was separated from the template by alkaline agarose gel electrophoresis as described [54]. This probe (25 kcpm) was co-precipitated with 70 µg total RNA, resuspended in 20 µl 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA by heating at 70°C for 30 min, and hybridized overnight at 30°C. Following addition of 10×mung bean nuclease buffer (Life Technologies) and 6 µg sonicated and denatured calf thymus DNA (Sigma), the products were digested with 0–80 U mung bean nuclease (Life Technologies) for 30 min at 30°C in a total volume of 300 µl. The reaction was stopped by adding 80 µl 4 M ammonium acetate, 20 mM EDTA, pH 8.0, 40 µg/ml yeast tRNA (Sigma) and ethanol precipitation. The protected products were resolved on a 6% polyacrylamide/urea sequencing gel and visualized by autoradiography. As size markers, T7 polymerase sequencing reactions were performed with the mHIFpex primer and pH13X8DH single-stranded DNA as template according to the instructions of the manufacturer (Pharmacia).

Cell culture. The mouse hepatoma cell line Hepa1 (also termed Hepa1c1c7) was a kind gift of L. Poellinger (Huddinge, Sweden). The mouse fibroblast cell line L929 (American Type Culture Collection [ATCC] CCL-1 NCTC clone 929) was a kind gift of V. O'Donnall (Bern, Switzerland). The human hepatoma cell line Hep3B (ATCC HB-8064) was obtained from ATCC. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Boehringer-Mannheim), 100 U/ml penicillin, 100 µg/ml streptomycin, 1×minimal essential medium non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate (all purchased from Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37°C. Oxygen tensions in the incubator (Forma Scientific, model 3319) were either 140 mm Hg (20% O_2 by vol., normoxia) or 7 mm Hg (1% O_2 by vol., hypoxia). The human epitheloid carcinoma cell line HeLaS3 (ATCC CCL-2.2) was obtained from ATCC and was cultured in suspension in Ham's F-12 medium (Life Technologies) supplemented as described above.

PCR analysis. Total RNA was isolated from normoxic and hypoxic Hepa1 and L929 cell cultures according to the method described by Chomczynski and Sacchi [55]. For cDNA synthesis, 6 μg RNA was heat denatured (3 min at 70°C) and reverse transcribed in 100 μl 50 mM Tris/Cl, pH 8.3, 60 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs and 1 U/μl RNAsin (Promega), using 5 μg $(dT)_{12-18}$ primers (Pharmacia) and 250 U Stratascript reverse transcriptase (RT) (Stratagene). Following incubation for 30 min at 37°C and for 30 min at 42°C, the reaction was stopped by heating to 95°C for 5 min. An aliquot (2 μl) of each cDNA reaction was subjected to PCR amplification using 50 pmol each of the forward primers mHIFexI.1 (5′-TTTCTGGGCAAACTGTTA-3′) and mHIFexI.2 (5′-CGCCTCTGGACTTGTCT-3′), and the reverse-primer mHIFexIII (5′-TAACCCCATGTATTTGTTC-3′) in 50 μl 1×PCR buffer (Stehelin), 0.2 mM dNTPs and 0.2 U SuperTaq DNA polymerase (Stehelin). Following 35 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 2 min on a Gene Amp System 9600 thermo-cycler (Perkin Elmer Cetus), the PCR products were analyzed by restriction digestion and Southern blotting.

Southern-blot analysis. Following gel electrophoresis of the PCR products through 1% agarose gels, the DNA was transferred to uncharged Biodyne A membranes (Pall) and cross-linked by ultraviolet irradiation (Stratalinker, Stratagene). The blots were hybridized to a gel isolated 5′ UTR probe homologous to the human HIF-1α cDNA [19], which was labeled to a specific radioactivity of 1×10^9 dpm/μg by the random-primed DNA-labeling method [52], or to the oligonucleotide mHIFpex (Fig. 3) which was 5′ end labeled (see above). Hybridization was performed in 6×NaCl/Cit (20×NaCl/Cit is 3 M NaCl, 0.3 M trisodium citrate, pH 7.0), 10×Denhardt's, 0.1% SDS, 1.1 mM $Na_4P_2O_7$, 17 mM Na_2HPO_4/NaH_2PO_4, pH 7.7, and 200 μg/ml sonicated salmon sperm DNA (Sigma) for 15 h at 65°C for DNA probes and at 60°C for the mHIFpex oligonucleotide. The blots were washed to a final stringency of 65°C in 0.1×NaCl/Cit, 0.2% SDS for DNA probes and of 60°C in 2×NaCl/Cit, 0.2% SDS for the mHIFpex oligonucleotide. Radioactive signals were recorded by phosphorimaging (Molecular Dynamics).

Reporter gene assays. Firefly luciferase reporter-gene constructs were obtained by inserting various fragments of the *Hif1a* exon I.1 upstream region into the promoterless luciferase vector pGL3Basic (Promega). A 1-kb *Pst*I−*Bst*XI fragment (*Pst*I cuts in the polylinker 5′ to the *Xba*I site shown in Fig. 3) was first subcloned into the *Pst*I−*Eco*RV sites of pBluescript (Stratagene). The resulting plasmid was then digested with *Hin*dIII together with either *Bam*HI (partially) or *Ssp*I, and the fragments subcloned into *Bgl*II−*Hin*dIII or *Sma*I−*Hin*dIII digested pGL3Basic, yielding the plasmids pGL1015Luc, pGL499Luc and pGL134Luc, respectively. For analysis of the putative HIF-1-binding site in the *Hif1a* promoter, the oligonucleotides mHIF5′ (5-AACTTACGTGGTTGCCTT-3′) or the mutant mHIF5′mt (5-AACTTAAAAGGTTGCCTT-3′) were annealed to the respective antisense oligonucleotides and inserted into the filled-in *Bam*HI site of the plasmid pGL3Promoter (Promega), which contains the luciferase gene driven by a heterologous simian virus 40 promoter. The copy number and orientation were determined using RVprimer4 (Promega) by T7-polymerase-mediated single-stranded DNA sequencing. Tissue culture cells (1×10^7 in 350 μl medium without fetal calf serum) were co-transfected with each 25 μg luciferase reporter gene construct and the β-galactosidase expression vector pCMVlacZ (kind gift of S. Kozlov, Zürich, Switzerland) by electroporation at 250 V and 960 μF (Gene Pulser, Bio-Rad). Thereafter, the cells were split in two aliquots and incubated for 24−30 h in 20% or 1% O_2, respectively. After washing twice with 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4 (NaCl/P_i), the cells were lysed in reporter lysis buffer (Promega) and luciferase and β-galactosidase activities were determined according to the instructions of the manufacturer (Promega) using a Biocounter M1500 luminometer (Lumac) and a DU-62 spectrophotometer (Beckmann), respectively. Differences in the transfection efficiencies and extract preparations were corrected by normalization to the corresponding β-galactosidase activities.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as described previously [53]. Briefly, 1×10^8 cells were washed twice with ice-cold NaCl/P_i and once with buffer A (10 mM Tris/HCl, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl), and lysed by 10 strokes of a Dounce homogenizer using a type-B pestle. The nuclei were pelleted and resuspended in buffer C (420 mM KCl, 20 mM Tris/HCl, pH 7.8, 1.5 mM $MgCl_2$, 20% glycerol) and incubated at 4°C for 30 min with gentle agitation. Immediately before use, buffers A and C were supplemented with 0.5 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of leupeptin, pepstatin and aprotinin, and 1 mM Na_3VO_4 (all obtained from Sigma). The nuclear extract was centrifuged and the supernatant was dialyzed twice against buffer D (20 mM Tris/HCl, pH 7.8, 100 mM KCl, 0.2 mM EDTA, 20% glycerol). Protein concentrations were determined using the Bradford protein assay (*Bio-Rad*) with bovine serum albumin as standard. For EMSAs, the mHIF5′ and mHIF5′mt oligonucleotides (see above), or an erythropoietin-derived HIF-1-binding oligonucleotide [53] were used as probes. All oligonucleotides were purified on 10% polyacrylamide gels prior to 5′ end-labeling of the sense strand as described above. Radiolabeled sense strands were annealed to a twofold molar excess of unlabeled antisense strands. DNA-protein binding reactions were carried out for 20 min at 4°C in a total volume of 20 μl containing 4−5 μg nuclear extract, 0.1−0.4 μg sonicated, denatured calf thymus DNA (Sigma) and 1×10^4 cpm oligonucleotide probe in 10 mM Tris/HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol and 5% glycerol and run on 4% non-denaturating polyacrylamide gels. Electrophoresis was performed at 200 V in 89 mM Tris, 89 mM boric acid, 5 mM EDTA (TBE buffer) at 4°C and dried gels were autoradiographed.

RESULTS

Isolation and characterization of the mouse *Hif1a* gene. We previously characterized the mouse cDNA for HIF-1α and found that it was encoded by an evolutionary conserved single-copy gene termed *Hif1a* [46]. For the molecular analysis of this gene, four rounds of screening of an λ phage library derived from an embryonic stem cell line (AB-1) were performed. In a first round, 1.3×10^6 λ plaques were screened using as probe a 0.5-kb fragment from the 5′ terminus of the human HIF-1α cDNA [26]. Seven positive clones were obtained and one of them (λH1) was subjected to restriction and sequence analysis. Comparison with the mouse cDNA sequence revealed that λH1 contained *Hif1a* exons II−VII (Fig. 1). To obtain the 3′ end of *Hif1a*, a second round of cloning was performed using a probe derived from the 3′ UTR of the HIF-1α cDNA. Following screening of 0.8×10^6 λ plaques of the same AB-1 library, six positive clones were isolated, one of which (λH25) contained exons VII−XV (Fig. 1). In a third round of cloning, an oligonucleotide termed mHIFpex derived from the alternative 5′ UTR (encoded by exon I.1) of the HIF-1α cDNA [46] was used as probe, resulting in a single positive clone (λH13). Finally, one clone (λH30) was obtained with a 5′ UTR mouse HIF-1α probe homologous to the human cDNA [19] (encoded by exon I.2). Inserts of the clones λH1 and λH25 were subcloned and se-

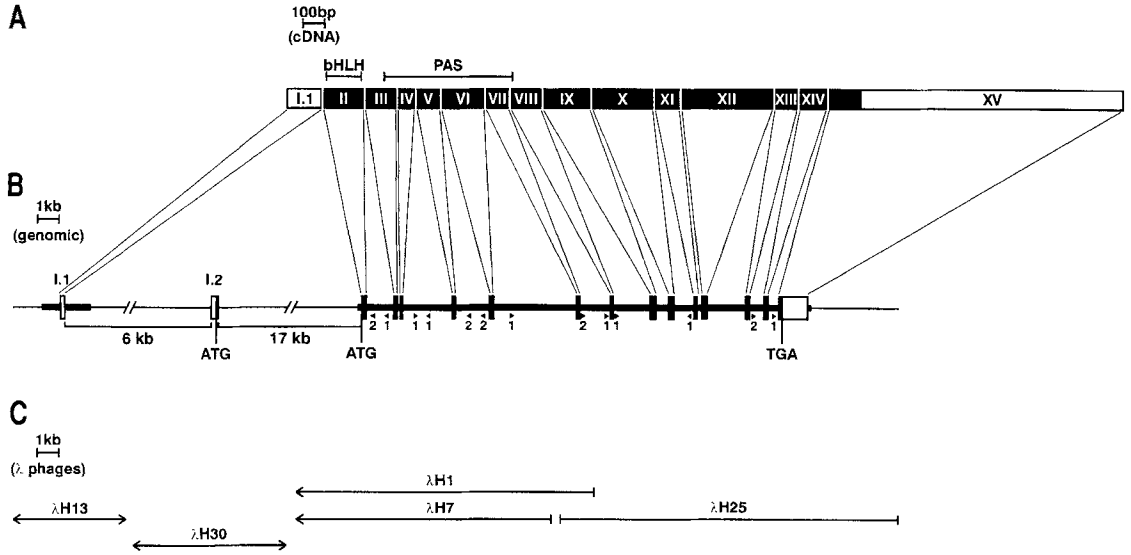

Fig. 1. Genomic organization of mouse *Hif1a*. (A) Structure of the mouse HIF-1α cDNA. Exons are numbered with roman numerals. The translated region is indicated by black boxes and the 5′ and 3′ UTRs by open boxes. The bHLH and PAS domains of HIF-1α are indicated. (B) Structure of the mouse *Hif1a* gene. Sequenced regions are indicated by the thick line and non-sequenced regions by the thin line. Filled and open boxes represent coding and non-coding regions, respectively. B1 and B2 mouse repetitive elements are indicated by arrowheads. (C) Location of the λ phage clones used to map and sequence the *Hif1a* gene. Note that the phage clone λH30 overlaps with λH13 and λH7.

exon no.	(length)	.....end	start...	intron no.	(length)	.....end	start...	exon no.	cDNA position
							TGCAA...	exon I.1	-28
exon I.1	(152 bp)	...TAAAG	GTAAG...	intron 1	(24 kb)	...AGTAG	GATGA...	exon II	124/125
exon II	(191 bp)	...TGCCG	GTGAG...	intron 2	(1241 bp)	...TGCAG	GTGGT...	exon III	315/316
exon III	(146 bp)	...CTCAG	GTAAA...	intron 3	(82 bp)	...TGCAG	TTTGA...	exon IV	461/462
exon IV	(85 bp)	...AAATG	GTGAG...	intron 4	(2357 bp)	...GTTAG	GCCCA...	exon V	546/547
exon V	(113 bp)	...GGAAG	GTACG...	intron 5	(1534 bp)	...TTCAG	GTGCT...	exon VI	659/660
exon VI	(203 bp)	...GAAAG	GTAAA...	intron 6	(3717 bp)	...AACAG	AATTA...	exon VII	862/863
exon VII	(107 bp)	...TGATA	GTAAG...	intron 7	(1347 bp)	...CCCAG	TGTTT...	exon VIII	969/970
exon VIII	(148 bp)	...GTAAG	GTAAG...	intron 8	(1727 bp)	...GACAG	TGGTA...	exon IX	1117/1118
exon IX	(221 bp)	...CGATG	GTCAG...	intron 9	(594 bp)	...TGCAG	ACACA...	exon X	1338/1339
exon X	(284 bp)	...CTGAG	GTTGG...	intron 10	(905 bp)	...CCCAG	CCTAA...	exon XI	1622/1623
exon XI	(123 bp)	...CTCAG	GTATG...	intron 11	(108 bp)	...GAAAG	GACAC...	exon XII	1745/1746
exon XII	(425 bp)	...CAAAG	GTATT...	intron 12	(1694 bp)	...TCCAG	AAATA...	exon XIII	2170/2171
exon XIII	(109 bp)	...GAATT	GTAAG...	intron 13	(726 bp)	...TTTAG	GGAAC...	exon XIV	2279/2280
exon XIV	(127 bp)	...CTCCG	GTTAG...	intron 14	(515 bp)	...TTCAG	ATTTA...	exon XV	2406/2407
exon XV	(1340 bp)	...AATAAACATCTTCTGTGGACCAGG-poly(A) addition site (end of exon XV)							3746

Fig. 2. Intron-exon splice junctions of the mouse *Hif1a* gene. The length of the exons and introns, sequences adjacent to intron-exon boundaries and the positions of the introns on the mouse HIF-1α cDNA sequence (numbering according to [46]) are indicated. The consensus splice donor and acceptor dinucleotides of each intron (GT...AG) are underlined and the polyadenylation signal is double underlined.

quenced, resulting in a total of 20463 bp which contained exons II–XV and the corresponding intervening sequences (Fig. 1). In addition, 2232 bp of λH13 containing exon I.1 were sequenced. Hybridization and restriction mapping analysis revealed that exons I.1 and I.2 are located approximately 24 kb and 17 kb, respectively, upstream of exon II (Fig. 1).

The *Hif1a* gene is composed of 15 exons separated by 14 introns (Figs 1 and 2). The lengths of the exons vary over 85–1340 bp. The longest, exon XV, contains the TGA stop codon and the 3′ UTR of HIF-1α, comprising around one third of the entire transcribed region. The poly(A) addition site is 18 bp downstream of the unique polyadenylation signal. The intron-exon boundaries all conform to the consensus GT and AG splice donor and acceptor dinucleotides [56]. A total of 13 B1 [57] and B2 [58] mouse repetitive elements were identified in the *Hif1a* introns (depicted as arrowheads in Fig. 1). In contrast to the regions flanking exon I.1, no low complexity repetitive elements longer than a few bases were detected.

A comparison with the 3746-bp mouse HIF-1α cDNA that was previously reported [46], revealed a total of 15 nucleotide substitutions; one in the 5′ UTR, nine in the coding region, and five in the 3′ UTR. Of the nine substitutions in the coding region, seven represent conservative changes and only two predicted amino acid changes. These amino acid changes are Ala116→Thr and Val773→Glu. The cDNA sequence reported by Li et al. [19], derived from the same Hepa1 cell line as our cDNA, spanned over the positions of 13 out of the 15 nucleotide substitutions. Seven of the changes that we found in the genomic DNA (including Ala116) were identical in our cDNA and in the cDNA reported by Li et al. [19] and, therefore, probably represent strain polymorphisms between *Hif1a* in the Hepa1 cell line (derived from mouse strain C57BL/6) and *Hif1a* in the AB-1

Fig. 3. Exon I.1 and flanking regions. The transcription initiation site mapped by mung bean nuclease protection (see Fig. 4) is indicated by a filled arrow, and the beginning of intron 1 by an open arrow. The sequence of exon I.1 is in bold. Restriction sites and low complexity repetitive elements are underlined, and putative transcription factor consensus binding sites are double underlined. The location of the oligonucleotide mHIFpex is depicted with a line over the sequence. A region of homology to a LINE-1 repetitive element is in italics, and an ORF potentially encoding a 14.1-kDa peptide is in bold.

embryonic stem cell line (derived from mouse strain 129Sv). The other six differences found in the genomic sequence (including Val773) were identical to the cDNA reported by Li et al. and, hence, might be the result of PCR, cloning and/or sequencing artifacts of our mouse HIF-1α cDNA. Other differences between Li's mouse HIF-1α cDNA and ours included a GC182→CG substitution and a 60-bp deletion in the 3′ UTR of Li's cDNA (data not shown). Sequencing of the genomic *Hif1a* clones, however, confirmed our cDNA sequence.

Characterization of *Hif1a* exon I.1. A 2232-bp *Xba*I fragment derived from the λ phage clone λH13 contained exon I.1 as well as 5′ and 3′ flanking sequences (Fig. 3). Nuclease protection assays were performed to map the 5′ end of exon I.1. Total RNA derived from Hepa1 cells was hybridized to an end-labeled, single-stranded probe of 523 nucleotides (Fig. 3, from the *Bam*HI site to the mHIFpex oligonucleotide). Excess single-stranded probe as well as protruding ends of the RNA-DNA hybrids were digested with increasing amounts of mung bean nuclease, and the products were separated on a sequencing gel along with sequencing reactions performed with the mHIFpex oligonucleotide as primer (Fig. 4). A single, strong band was repeatedly found, whose intensity decreased with increasing amounts of mung bean nuclease. Multiple, much weaker signals were also observed, but these bands were not digested with mung bean nuclease in a dose-dependent manner and hence probably result from unspecific cleavage. No bands were found in the untreated control lane, indicating that the observed pattern is not due to unspecific probe fragmentation. The unique protected fragment corresponds to the T at position 903 of the sequence (Fig. 3), 2-bp downstream of a putative CA cap signal. In support of this finding, a T located in the prefered region centered 2-bp downstream of the cap signal has been detected in 131 of 303 cases [59]. These data give rise to a length of 152 bp for exon I.1, 29 bp longer than previously found by the 5′ rapid amplification of cDNA ends (RACE) technique [46]. 5′ RACE is a modification of the primer-extension analysis of mRNA cap sites and thus provides a complementary method to nuclease protection. The slightly shorter 5′ RACE product might be attributed to premature first strand synthesis termination.

An ORF was identified in exon I.1 which did not contain an ATG translation initiation codon and was not in-frame with the ORF of exons II−XV (data not shown). Consistent with the genomic sequence, the ATG beginning with the second nucleotide of exon II was predicted as the translational start site on mRNA transcripts containing the 5′ UTR encoded by exon I.1 [46]. No ATG codon is found at this position in the human HIF-1α cDNA [26] whose translational start site corresponds to the exon I.2-derived ATG present on the mouse cDNA sequence reported by Li et al. [19]. This suggests that the organization of the 5′ regions differs between the two species. Thus, the use of exon I.2 results in a *Hif1a* gene product that is 12-amino-acids longer than the predicted gene product derived from exon I.1. The shorter protein, however, still begins four amino acids before the bHLH domain (Fig. 1). The predicted translational start site (the actual N-terminus of the HIF-1α protein has not yet been determined) on exon I.2 is TTCGCCATGG, and conforms better to the consensus translation initiation site GCCRCCATGG [60] than the initiation site on exon II (TAAAGGATGA), predicted to be used if exon I.1 encodes the 5′ UTR of HIF-1α. The second most critical position (G at +4), present in 46% of vertebrate mRNAs [60], was found only in the exon I.2 start

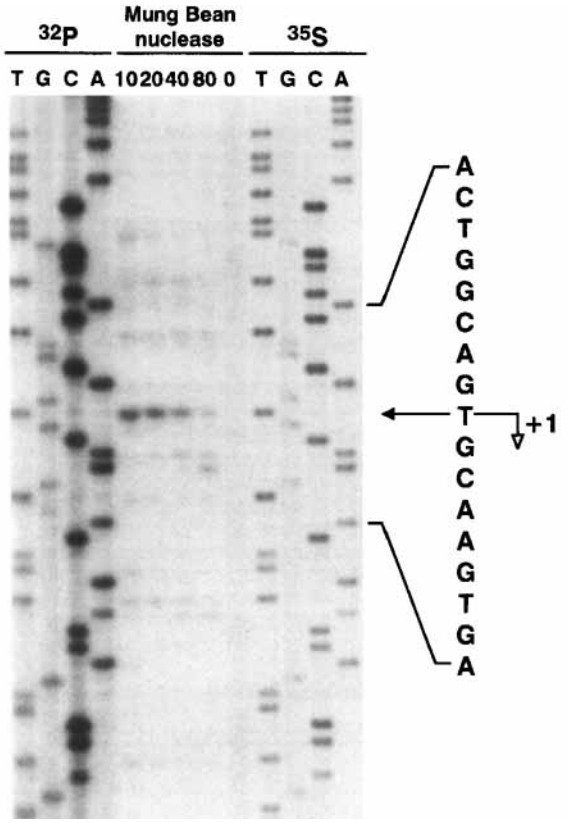

Fig. 4. Mung bean nuclease protection mapping of the *Hif1a* exon I.1 5′ end. To identify the transcriptional initiation site of *Hif1a* exon I.1, total RNA derived from mouse Hepa1 cells was hybridized to a single-stranded probe prepared using the 5′ end-labeled oligonucleotide mHIFpex (see Fig. 3) as primer for a Klenow extension reaction. Following treatment of RNA-DNA hybrids with the indicated amount of mung bean nuclease, the protected fragments were separated on a sequencing gel and visualized by autoradiography. Sequencing reactions using either the [γ-^{32}P]ATP end-labelled mHIFpex primer or unlabeled mHIFpex primer with [^{35}S]dATP[αS] incorporation served as markers. The position of the unique protected band is indicated by an arrow.

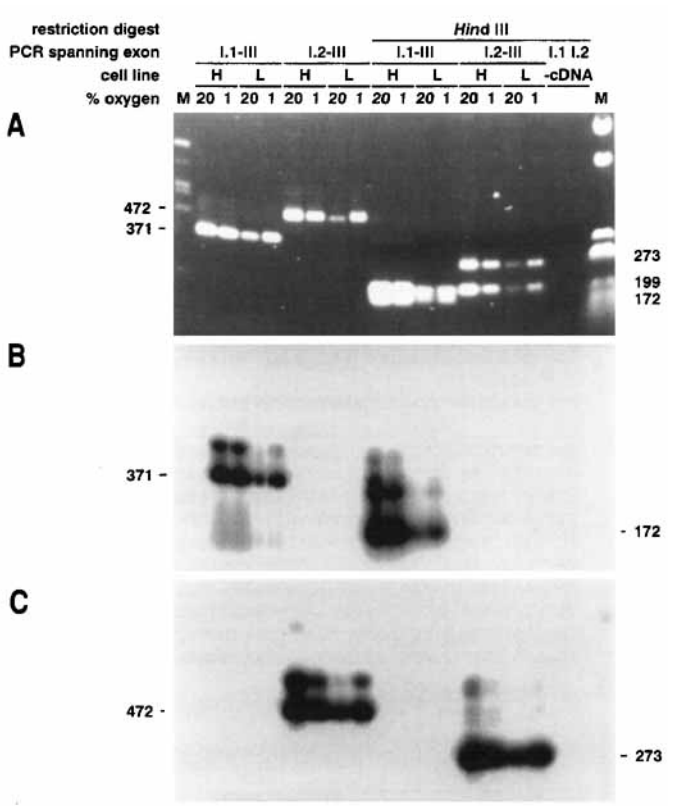

Fig. 5. Co-expression of two different HIF-1α mRNA transcripts in normoxic and hypoxic Hepa1 and L929 cells. (A) RT-PCR analysis of total RNA derived from normoxic (20% O_2) and hypoxic (4 h at 1% O_2) Hepa1 hepatoma (H) and L929 fibroblast (L) cells. Primer pairs spanning either exons I.1-III or exons I.2-III were used, and half of the RT-PCR products were digested with *Hind*III which cuts in exon II. An ethidium-bromide-stained agarose gel is shown with marker lanes (M) on both sides. The lengths of the RT-PCR products are indicated. -cDNA, control PCR lacking template cDNA. (B) Southern-blot analysis of the RT-PCR products using an exon I.1-specific hybridization probe. (C) Southern-blot analysis of the RT-PCR products using an exon-I.2-specific hybridization probe.

site. However, the most critical purine at position −3, which is conserved in 97% of vertebrate mRNAs [60], is present in both 5′ UTRs. Therefore, initiation of translation may not be severely affected.

Co-expression of endogenous mouse HIF-1α transcripts which contain 5′ regions derived from either exon I.1 or exon I.2. To further investigate the expression of the two mouse HIF-1α mRNA isoforms, we established an RT-PCR assay using forward primers which are specific for either exon I.1 or exon I.2, and a reverse primer located on exon III. To confirm the specificity of the PCR, the products were digested with *Hind*III, which cuts only in exon II, and visualized by agarose gel electrophoresis and ethidium bromide staining (Fig. 5A). Total RNA was isolated from normoxic (20% O_2) or hypoxic (4 h at 1% O_2) Hepa1 mouse hepatoma or L929 mouse fibroblast cells. We have previously shown that VEGF mRNA is markedly induced in these total RNA preparations, and that HIF-1 DNA-binding activity was detectable exclusively in nuclear extracts derived from parallel hypoxic but not normoxic cell cultures [47]. In contrast, Northern-blot estimations revealed that HIF-1α mRNA levels were not significantly induced in these RNA preparations [47].

RT-PCR analysis of the Hepa-1 and L929 cell lines using the primer pairs I.1-III and I.2-III revealed single products of the expected length which displayed the correct restriction pattern following *Hind*III digestion (Fig. 5A). Assuming that a single promoter drives the expression of a premature transcript containing exon I.1 followed by exon I.2, one would expect an additional band in the I.1-III PCR of equal intensity as in the I.2-III PCR. However, such a larger band of comparable strength could not be observed (Fig. 5A), suggesting that the two transcripts are independently regulated by their own promoters. Our conclusion is confirmed by the finding that neither our laboratory nor another group (A. Damert, personal communication) found (by 5′ RACE) HIF-1α mRNA transcripts that extended beyond the 5′ end of either exon I.1 or exon I.2. No differential expression of the two mRNA isoforms were observed in the two cell lines or due to the two oxygen concentrations. Competitve PCR using all three primers in a single reaction suggested that exon I.1 might be expressed at least 10-fold more efficiently than exon I.2. However, since exon I.2 is highly G+C rich, we cannot exclude the possibility that differences in the PCR efficiency rather than different expression levels led to this result (data not shown).

These RT-PCR products were further analyzed by Southern blotting and hybridization to probes which are specific for exon I.1 (Fig. 5B) and exon I.2 (Fig. 5C), respectively. The observed specific hybridization signals confirmed the identity of the RT-PCR products. Intriguingly, in addition to the expected PCR

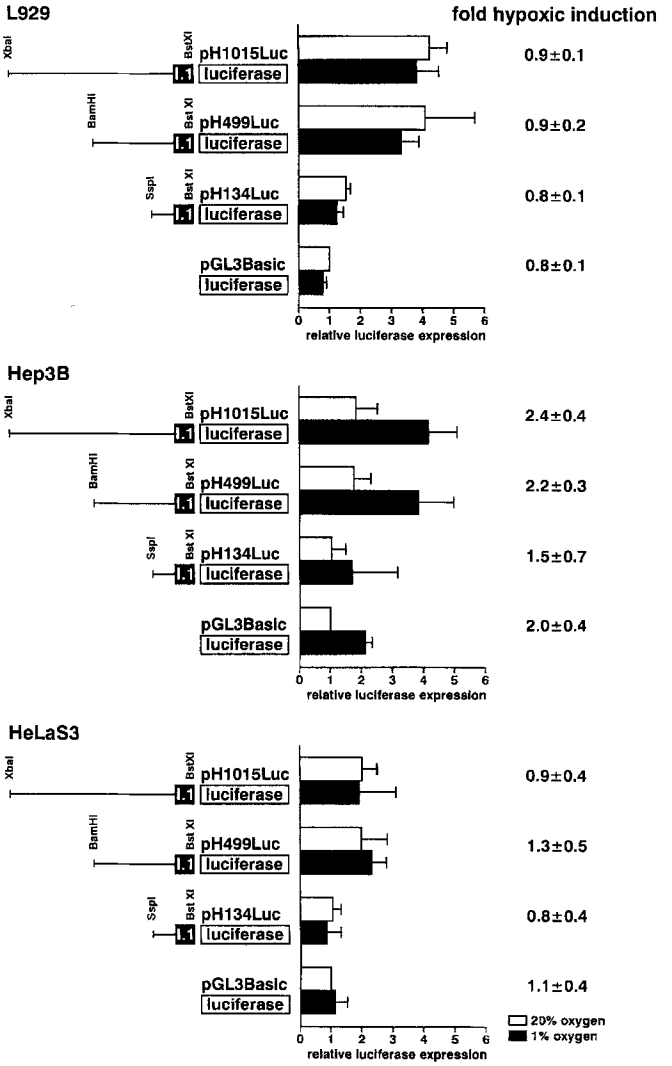

Fig. 6. Reporter gene expression driven by the *Hif1a* exon I.1 promoter. Luciferase reporter gene activity following transient transfection and hypoxic induction of L929, Hep3B and HeLaS3 cells with luciferase expression plasmids containing various fragments of the *Hif1a* exon I.1 5′ flanking region as indicated. A co-transfected β-galactosidase expression vector served as internal control for transfection efficiency and extract preparation. All values were normalized to the normoxic luciferase activities obtained with the empty vector pGL3Basic which were arbitrarily defined as 1. Means ± SD of three independent experiments are shown.

product, a weaker band of higher molecular mass appeared with both primer pairs. In principle, a fraction of the RT-PCR products would be expected to be longer if they result from a differentially spliced common precursor mRNA and thus contains both possible first exons. However, such a longer RT-PCR product could occur only in one of the two RT-PCR products and, even more importantly, the hybridization probes would cross-hybridize with such a product. Since this was not the case, it was concluded that the larger hybridizing RT-PCR products represent either PCR artifacts or that an additional, differentially spliced exon is present downstream of exons I.1 and I.2. These results suggest that mouse HIF-1α mRNA is co-expressed as two transcripts containing different 5′ UTRs and ATG translation initiation codons, and that *Hif1a* gene expression is driven by two different promoters which do not seem to be differentially regulated in hepatoma cells compared to fibroblast cells or during hypoxia compared to normoxia.

Structural analysis of the flanking regions of exon I.1. 0.9 kb of the 5′ flanking region and 1.2 kb of the 3′ flanking region (intron 1) of exon I.1 were sequenced (Fig. 3). A computer-assisted search using the consensus DNA-recognition-site-containing tfsites.dat database of the GCG program package [61] revealed a number of perfect matches to consensus sites including a Sp1 site, a TATA box and an AP-1 site (Fig. 3). The TATA box is located 300 bp upstream of the transcription initiation site and hence does not conform to the typical location of TATA boxes which are preferably found 20–36 bp upstream of the transcriptional start site [59]. However, a non-canonical TATA box (TATTAT) was located in the expected position 23 bp upstream of the beginning of exon I.1. Interestingly, a putative HIF-1-binding site (TACGTGGT) was identified 82 bp upstream of the exon I.1 start site. This site perfectly matched the consensus HIF-1-binding site BACGTGSK [18]. Apart from this putative HIF-1 site in the *Hif1a* exon I.1 promoter, only one additional match to the HIF-1 consensus binding site was detected in the 20.4-kb sequenced part of the *Hif1a* gene; the motif GACGTGCT was found in exon VI, corresponding to nucleotide positions 740–747 in the mouse HIF-1α cDNA sequence [46]. However, neither of these two specific 8-bp HIF-1 motifs have so far been found in other genes.

In contrast to the introns 2–14, no B1 and B2 mouse repetitive elements could be identified in the sequences flanking exon I.1. In contrast, a region of 212 bp sharing 74% similarity with a LINE-1 repetitive element (L1MdA11) [62], which has not been found in introns 2–14, was localized 364–152 bp upstream of the transcription initiation site of exon I.1 (Fig. 3). Intriguingly, this element was 67% similar to a 146-bp stretch in the 3′ UTR of the AhR gene [63]. The biological significance of this finding, however, awaits further investigations. The *Hif1a* exon I.1 flanking regions were enriched in low complexity repetitive elements such as $(A)_n$, $(T)_n$, $(GT)_n$, $(GA)_n$ and $(ATCN)_n$ repeats (underlined in Fig. 3). Interestingly, the $(ATCN)_n$ repeat was located within an ORF (boldface in Fig. 3) which potentially encodes a 14.1-kDa peptide. These repeats are concentrated in the sex-determining region of the mouse Y chromosome and have been proposed to encode primordial proteins [64, 65]. mRNA containing such 4-bp repeats (also termed GATA repeats for the complementary strand) is sometimes transcribed [64, 65], but whether the $(ATCN)_n$ element in the *Hif1a* exon I.1 promoter is also transcribed is unknown.

Functional analysis of the 5′ flanking region of exon I.1. To examine the promoter activity of the 5′ flanking region of exon I.1, several cell lines were transfected with luciferase expression constructs that contained exon I.1 flanking sequences of various length. These constructs were transiently expressed in L929, Hep3B and HeLaS3 cells cultured under normoxic (20% O_2) or hypoxic (1% O_2) conditions for 24–30 h. A co-transfected β-galactosidase expression vector was used to correct for differences in transfection efficiency and extract preparation. The ratios between luciferase and β-galactosidase activities were normalized to the normoxic value obtained with the empty vector pGL3Basic which was arbitrarily defined as 1. In normoxia, the 1015-bp *Xba*I–*Bst*XI and the 499-bp *Bam*HI–*Bst*XI fragments stimulated reporter gene expression about fourfold in mouse L929 cells and about twofold in human Hep3B and HeLaS3 cells (Fig. 6). The 134-bp *Ssp*I–*Bst*XI fragment only slightly stimulated luciferase expression in L929 cells, but was indistinguishable from the promoterless parental vector in Hep3B and HeLaS3 cells. These results suggest that the 0.4-kb exon I.1 upstream region functions as a minimal promoter whose activity cannot be enhanced by additional 0.5-kb 5′ sequences. Hypoxic incubation did not lead to a significant induction of reporter gene

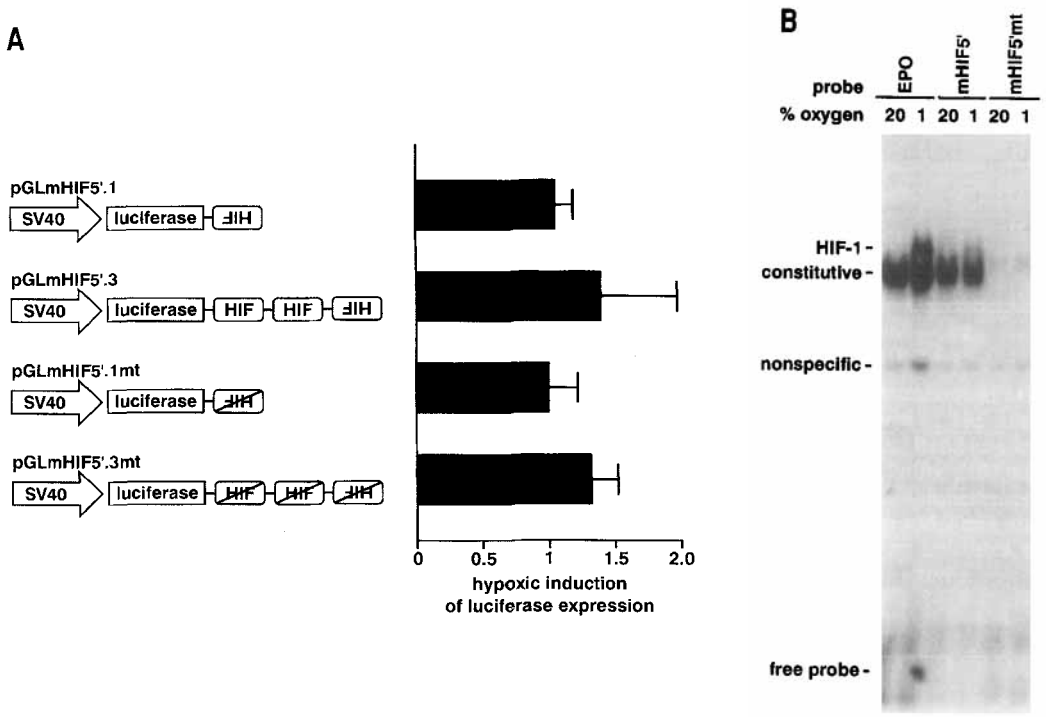

Fig. 7. Lack of enhancer function and DNA-binding activity of the putative HIF-1-binding site in the *Hif1a* exon I.1 promoter. (A) Reporter gene expression of simian virus 40 (SV40) promoter-driven luciferase constructs containing wild-type (mHIF5′) or mutant (mHIF5′mt) putative HIF-1-binding sites derived from the *Hif1a* exon I.1 promoter. One or three copies of an 18-bp oligonucleotide were inserted downstream of the luciferase gene as indicated. Reporter gene activity was determined following transient transfection into Hep3B cells as described in Fig. 6. Means ± SD of three independent experiments are shown. (B) EMSA using nuclear extracts derived from normoxic or hypoxic (4 h, 1% O_2) Hepa1 cell cultures. Oligonucleotides derived from the erythropoietin 3′ hypoxia-inducible enhancer or the *Hif1a* exon I.1 5′ flanking region (wild-type mHIF5′ and mutant mHIF5′mt) were used as probes.

expression. The twofold increase observed in hypoxic Hep3B cells, but not in the other cell lines, was also detected with the empty vector alone and hence cannot be attributed to the function of the *Hif1a* exon I.1 promoter.

The putative HIF-1-binding site in the *Hif1a* exon I.1 5′ flanking region is not functional. One possible explanation for the failure of the exon I.1 promoter to hypoxically induce reporter gene expression despite the presence of a putative HIF-1-binding site might be the presence of negative regulatory sequences on the same plasmid construct. Therefore, an 18-bp oligonucleotide was analysed, encompassing this putative HIF-1 site, which was inserted in one or three copies downstream of the luciferase gene driven by a heterologous simian virus 40 promoter. As a control, analogous plasmids were constructed containing a mutated version of these oligonucleotides (see Materials and Methods). In previous experiments, a similar approach using three copies of the HIF-1-binding site derived from the erythropoietin gene 3′ flanking region [43, 53], or a single copy of two tandemly repeated HIF-1-binding sites derived from the transferrin gene (Rolfs, A., Kvietikova, I., Gassmann, M. and Wenger, R. H., unpublished results), resulted in a marked induction of reporter gene expression after hypoxic exposure. In contrast (Fig. 7A), the *Hif1a* promoter-derived putative HIF-1-binding site failed to convey hypoxic induction to reporter gene expression. In parallel experiments, the HIF-1-binding sites derived from the transferrin gene enhanced reporter gene expression 9.3-fold, indicating that the cells were hypoxically stimulated (data not shown).

To answer the question of whether the HIF-1 complex is capable of binding to the *Hif1a*-derived putative HIF-1 site, EMSAs were perfomed using nuclear extracts derived from normoxic and hypoxic Hepa1 cells. Whereas the HIF-1 DNA-binding activity in these extracts was readily detectable with an erythropoietin-derived HIF-1 oligonucleotide probe, almost no binding to the *Hif1a*-derived probe (mHIF5′) was observed (Fig. 7B). In contrast, the constitutive DNA-binding activity, previously attributed to ATF-1/CREB-1 function [53], remained unaffected with the *Hif1a*-derived wild-type probe and disappeared only with the mutant oligonucleotide mHIF5′mt (Fig. 7B). Similar results were obtained with the putative HIF-1-binding site located in exon VI (data not shown). These results demonstrate that the putative HIF-1-binding site in the exon I.1 promoter is not functional.

DISCUSSION

Prior to the present report, the structural organization of genes encoding mammalian members of the bHLH-PAS family was only known for the mouse AhR gene (*Ahr*) [66]. The architecture of the *Hif1a* and *Ahr* genes shares some similarities; both genes contain relatively long 5′ introns, the bHLH and PAS domains are encoded by different exons, and the last exons, by far the longest in both genes, code for the entire 3′ UTRs. Such as *Hif1a*, the *Drosophila sim* and *per* genes also contain non-coding first exons which are separated by relatively long introns from the ATG-containing exon [67, 68]. In a survey of 699 vertebrate mRNAs, nearly 25% of the examined mRNAs have an intron between the promoter and the ORF [60]. This finding has two important putative consequences: (a) the first intron might bear transcriptional regulatory elements: (b) the promoter may be switched in response to tissue-specific signals, thereby replacing a weak by a strong promoter and/or exchanging an inef-

ficiently translated 5′ UTR for one that appears more favourable [60].

In this work we have shown that 0.9 kb of *Hif1a* exon I.1 5′ flanking sequences display only moderate promoter activity, probably because the high density of repetitive elements present in this region reduces the number of possible transcription-factor-binding sites. Thus, additional *cis*-regulatory elements might be required for efficient transcriptional activation. In support of this hypothesis, an exogenous simian virus 40 enhancer induced the activity of the 499-bp exon I.1 promoter 2.8 ± 0.4-fold (data not shown). Additional work will be necessary to identify and locate this enhancer in the *Hif1a* gene. It is interesting to note that while HIF-1α mRNA is ubiquitously transcribed, some tissue-specific variations in the expression levels can nevertheless be observed [40, 45–47]. Exon I.1-specific and I.2-specific probes will be required to examine the possibility of differential tissue-specific, as well as developmental and conditional, expression of the two different HIF-1α mRNA isoforms. Assuming that this is the case, it will be interesting to identify the responsible regulatory elements which might be located in the 5′ flanking region as well as in the first intron.

With regard to our previous finding that HIF-1α is not hypoxically regulated at the mRNA level [40, 46, 47] while HIF-1α protein content is drastically increased [26, 41, 44], it seems likely that regulation of HIF-1α expression takes place at the translational and/or post-translational level(s). Indeed, a recent report suggested that hypoxia increases the protein stability of HIF-1 [48]. Moreover, the presence of two different 5′ UTRs in the HIF-1α mRNA population offers the possibility that HIF-1α expression is dependent on the efficiency with which the two mRNA species are translated. Interestingly, while the exon-I.1-derived 5′ UTR contains a moderate G+C fraction (49%), the exon-I.2-derived 5′ UTR is G+C-rich (73%) and displays comparable CpG and GpC dinucleotide frequencies. It therefore fulfills the criteria of CpG islands, known to be associated with constitutively active promoters of housekeeping genes often overlapping with the 5′ UTRs [69]. Moreover, G+C-rich 5′ UTRs are often poorly translated [60] and might be implicated in post-transcriptional regulation [69]. Therefore, it is possible that the translation rate of mouse HIF-1α is dependent on the ratio between the two mRNA isoforms and, hence, on the relative activity of the two *Hif1a* promoters. The functional activity of HIF-1α might also be altered due to the lack of the 12 N-terminal amino acids in the exon-I.1-derived isoform.

One intriguing feature of the *Hif1a* exon I.1 promoter is the presence of a putative HIF-1-binding site 82 bp upstream of the transcription initiation site. In addition, a putative AP-1 site is located 28 bp downstream of the HIF-1 site. This configuration is reminiscent of the VEGF promoter where a HIF-1-binding site has been structurally and functionally identified approximately 1 kb upstream of the VEGF transcriptional start site [15, 16, 18]. Interestingly, an AP-1 site is also located 30 bp downstream of the VEGF HIF-1 site, a configuration which is conserved between human, rat and mouse VEGF promoters. However, whereas this fragment conveys oxygen-regulated expression to a reporter gene, the *Hif1a*-derived putative HIF-1-binding site is not hypoxia responsive. This might be explained in part by the marked reduction in HIF-1 DNA binding to this site, probably due to a mismatch at the last position of the 8-bp *Hif1a* putative HIF-1 site compared to the VEGF HIF-1 site. This position, however, still conforms to the 8-bp tentative consensus HIF-1 site [18]. In addition, a CACAG element located 5 bp downstream of the HIF-1 site in the erythropoietin [14] and VEGF [15, 16, 18] genes, which is absolutely required for full hypoxic enhancer activity, is absent in the *Hif1a* gene. Together, these two observations provide an explanation for the lack of oxygen-responsiveness of the *Hif1a* exon I.1 promoter despite the presence of a HIF-1 consensus binding site.

It will be interesting to determine whether an exon I.1 homolog is also expressed in human tissues, and, following cloning, to structurally and functionally analyze the mouse and human promoters of exon I.2. Sequence comparisons between the two species will help to identify conserved regulatory elements and will provide further information on the mechanisms of constitutive, tissue-specific and isoform-specific regulation of HIF-1α mRNA expression. Even more importantly, since it is now clear that HIF-1α is expressed as two mRNA isoforms in the mouse, it will be insightful to examine the possibility of differential, isoform-specific, post-transcriptional mechanisms of hypoxic HIF-1α activation.

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